

How a growing organismal perspective is adding new depth to integrative studies of morphological evolution

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ABSTRACT

Over the past half century, the field of Evolutionary Developmental Biology, or Evo-devo, has integrated diverse fields of biology into a more synthetic understanding of morphological diversity. This has resulted in numerous insights into how development can evolve and reciprocally influence morphological evolution, as well as generated several novel theoretical areas. Although comparative by default, there remains a great gap in our understanding of adaptive morphological diversification and how developmental mechanisms influence the shape and pattern of phenotypic variation. Herein we highlight areas of research that are in the process of filling this void, and areas, if investigated more fully, that will add new insights into the diversification of morphology. At the centre of our discussion is an explicit awareness of organismal biology. Here we discuss an organismal framework that is supported by three distinct pillars. First, there is a need for Evo-devo to adopt a high-resolution phylogenetic approach in the study of morphological variation and its developmental underpinnings. Secondly, we propose that to understand the dynamic nature of morphological evolution, investigators need to give more explicit attention to the processes that generate evolutionarily relevant variation at the population level. Finally, we emphasize the need to address more thoroughly the processes that structure variation at micro- and macroevolutionary scales including modularity, morphological integration, constraint, and plasticity. We illustrate the power of these three pillars using numerous examples from both invertebrates and vertebrates to emphasize that many of these approaches are already present within the field, but have yet to be formally integrated into many research programs. We feel that the most exciting new insights will come where the traditional experimental approaches to Evo-devo are integrated more thoroughly with the principles of this organismal framework.

Key words: integrative organismal biology, plasticity, Evo-devo, model clade, Eco-evo-devo, constraints.

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I. INTRODUCTION

Over the last decade evolutionary-developmental biology, or Evo-devo, has grown into a dynamic field that rests at the interface of many biological disciplines beyond its founding pair (Haag & Lenski, 2011; Laland *et al.*, 2015; Moczek *et al.*, 2015). With its increasingly sprawling reach, Evo-devo has both adopted perspectives from and provided new insights to disciplines as distant as neurobiology, medicine, genetics, ecology, and agriculture among others. Although the molecular genetic backbone of Evo-devo emerged from the traditional model organisms (e.g. mouse, chick, zebrafish, fruit fly, nematode, frog, *Arabidopsis*), an increasingly large number of new species are being used in comparative studies of development (Abzhanov *et al.*, 2008; Jeffery, 2008; Bolker, 2014; Braasch *et al.*, 2015; Specht & Howarth, 2015; Herron, 2016). These new species are providing fresh insights into the evolutionary mechanisms of morphological diversification. This expanding focus on non-traditional organisms has made great progress towards elucidating the evolutionary processes facilitating or constraining morphological diversification and has added finer resolution to our understanding of the evolution of development. Ironically, there have been coincident discussions of strategically limiting the number of species used in Evo-devo studies to fit into the predefined research areas (Jenner & Wills, 2007; Sommer, 2009), which results in a patchwork of distantly related taxa seemingly selected for a pragmatic combination of limited scope (opposed by Brigandt & Love, 2010). This argument is antithetical to the way most evolutionary studies are conducted. In fact, it has been argued that there is a dearth of evolutionary analyses within modern Evo-devo (Diogo, 2016, 2018). We feel strongly that the synergistic approach of employing experimental model species and comparative analysis of model clades is where the most novel insights will emerge in the coming years. As we argue here, in order to understand what factors control evolutionary patterns and rates of morphological diversification, it is essential to incorporate integrative organismal biology more thoroughly into the conceptual framework of Evo-devo.

II. AN ORGANISMAL PERSPECTIVE TO THE DEVELOPMENTAL BASES OF MORPHOLOGICAL DIVERSITY

There have been repeated calls for a more integrated approach to the comparative study of development (Brakefield, 2003; Wake, 2008; Gilbert & Epel, 2010; Laland *et al.*, 2015; Sommer & Mayer, 2015). In fact, one of the field's greatest strengths is its fluid definition of 'integrative' (Wake, 2015). However, we feel that within Evo-devo, ecology and organismal life history must be more than backdrops for the stage upon which developmental stories are told. Phylogenies should be used to create *a priori* hypotheses of morphological evolution and its associated developmental causation. The evolution of morphological form should not be restricted

to atomizing morphological structures and their underlying developmental-genetic mechanisms during morphogenesis but should also account for the development and evolution of co-varying traits throughout the whole of ontogeny. At the level of the individual, the organismal framework for Evo-devo reminds us of the complex interplay of molecular, physical, and environmental factors that shape organismal form. At a broader evolutionary level, this conceptual framework reminds us of the interaction between intrinsic and extrinsic factors that shape the process of morphological diversification. As highlighted herein, this perspective is readily present within Evo-devo and has, at times, been paired successfully with the more traditional experimental approaches to Evo-devo. However, its distinct explanatory power towards the evolutionary processes of morphological diversification has rarely been formally discussed.

Supporting the objectives of the organismal framework are three pillars. The first pillar emphasizes the necessity for formal analysis of organismal diversity within the appropriate evolutionary context, where the field moves beyond discussion of developing new model species and towards the adoption of new 'model clades'. The second pillar stresses the need for further commitment towards uncovering the developmental origins of evolutionarily relevant variation, particularly among closely related populations and species. The final pillar emphasizes the need to dissect the complex, higher order processes that shape patterns of phenotypic variation. As illustrated in the examples used below, each of these pillars is present in organismal biology, but they are not always given the depth of discussion they deserve. We hope that by formalizing these issues they may become more central to future discussions of morphological diversification.

(1) The first pillar: the explanatory power of model clades over model species

Among the many developmental aspects embraced by the molecular renaissance of Evo-devo, the discovery of the deep conservation of the genetic toolkit, developmental and anatomical modularity, and the importance of *cis*-regulation are arguably of the greatest significance (Müller, 2007; Carroll, 2008). Nevertheless, these foundations were built with distantly related model species and, therefore, lacked the phylogenetic resolution needed truly to understand the origin, innovation and elaboration of morphological form (Fig. 1). This traditional approach to Evo-devo followed directly from work in developmental biology, taking advantage of the genetic tools available to drill deep into the developmental regulation of a particular trait. This approach, however, did not investigate the vast majority of morphological diversity and remains blind to the transitional evolutionary steps that led to those extremes (Fig. 1). Because of the phylogenetic distances between model species, the reconstruction of ancestral states should be interpreted with caution.

The fields of Ecology and Evolutionary Biology have long studied diverse adaptive radiations to understand better the selective factors that have shaped the patterns of species and morphological diversity (e.g. Schluter, 2000; Grant &

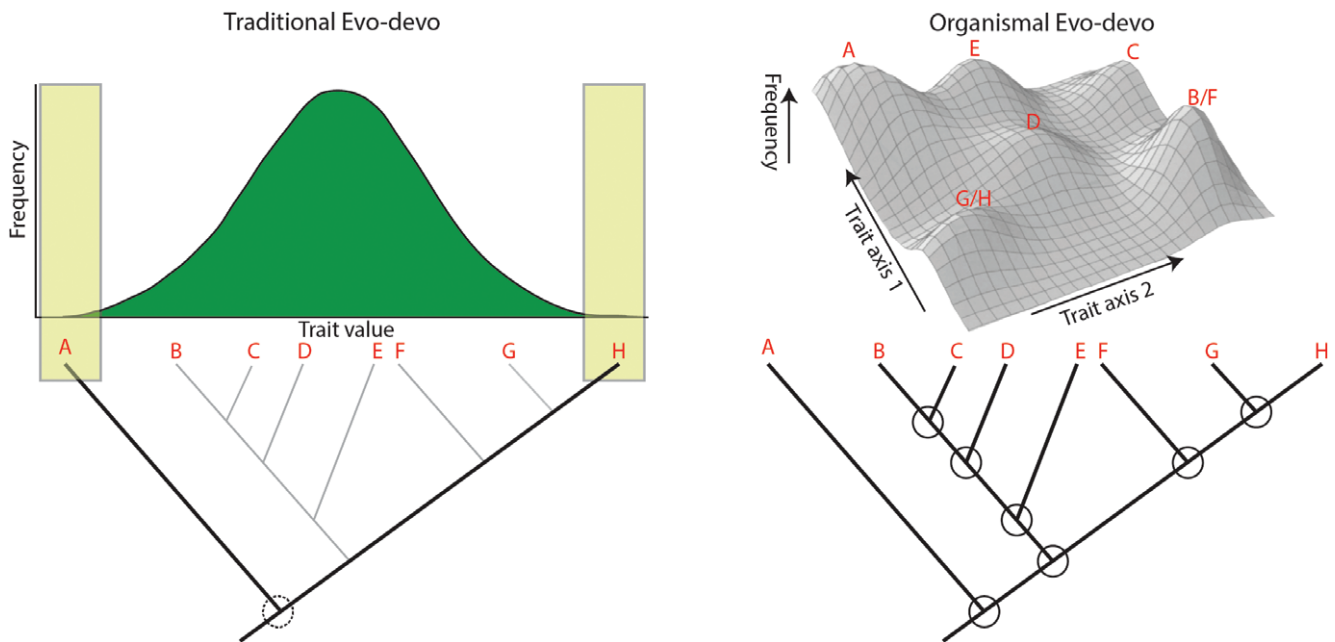


Fig. 1. Summarizing the differences between the traditional approach to Evo-devo and the organismal framework that we discuss herein. The traditional framework typically uses distantly related model species that have rich experimental toolkits. These species often lie at the extremes of the morphological variation observed in a clade (yellow boxes). This, however, reduces the phylogenetic resolution of the analyses and ignores the majority of the morphological variation exhibited by a clade (green distribution). By contrast, the organismal framework uses a high-resolution map of developmental and anatomical variation accounting for all evolutionary relevant variation exhibited for the traits of interest within a clade. Within the organismal framework investigators have a stronger ability to reconstruct ancestral character states (node circles).

Grant, 2008; Losos, 2009). Although the use of satellite species – species thought of as revolving around a traditional model species – has had some success in Evo-devo, their utility remains limited to few systems (Simpson, 2002; Jenner & Wills, 2007; Alfred *et al.*, 2015). We argue that a high-resolution comparative approach to Evo-devo, more comparable to the study of adaptive radiations, will have far greater explanatory power than the traditional approach to Evo-devo primarily relying on distantly related model systems (Fig. 1).

Through the rigorous use of phylogenetic comparative methods (Pennell & Harmon, 2013), investigators can test whether a change in development occurs at the same node as the origin of a trait, later during its diversification, or if developmental processes have evolved independent of the change in morphology. The explicit use of phylogenetic comparative methods will help reconcile the alternative evolutionary scenarios of (i) developmental systems drift where development changes as a result of relaxed selection (True & Haag, 2001; Kiontke *et al.*, 2007); (ii) development changing because of selection acting directly on developmental processes; or (iii) changes that are directly associated with the modification of an overlying structure during an ecological transition. A high-resolution phylogenetic map of developmental processes at and above the level of gene function will allow Evo-devo investigators to test these alternatives and move beyond the telling of ‘just-so’ evolutionary tales.

Because all of life has evolved from a sequence of ancestors, every species has its own unique history. Simply due to ancestry, clades are predisposed to certain biases and limitations in what they are capable of. Therefore, closely related species may be predicted to respond to similar selection pressures using similar developmental mechanisms more often than more distantly related species (Vavilov, 1922; Futuyma, Keese & Scheffer, 1993; Schluter, 1996; Losos, 2010; Sanger, *et al.*, 2012a; Stern, 2013). We may expect this continuity of information to weaken over time or after major evolutionary transitions as an ancestral signature becomes overwritten.

Dissecting the developmental basis of convergent phenotypes has been central to the growth of Evo-devo over the last 20 years (e.g. Sucena & Stern, 2000; Yoon & Baum, 2004; Colosimo *et al.*, 2005; Shapiro, Bell & Kingsley, 2006; Stern, 2013). With the more formal adoption of phylogeny and comparative methods Evo-devo may provide new insights into the developmental and genetic bases of convergent phenotypes, providing additional insights into whether convergent phenotypes arise from similar or distinct developmental processes. This approach promises to address why some clades show extensive parallel evolution while others primarily show convergence. The devil of this perennial question likely lies in the details of the evolutionary processes producing and structuring variation across evolutionary scales, which can only be uncovered with the appropriate level of phylogenetic resolution.

Sanger *et al.* (2013) described a convergent pattern of craniofacial dimorphism between two distantly related lineages of *Anolis* lizards. Despite few similarities in their ecology, males in both lineages evolved remarkably longer faces than their conspecific females. Strikingly, this was accomplished using distinct developmental strategies. One clade, the *hendersoni* clade, appears to have recruited a widespread, likely ancestral, strategy to reach its extreme dimorphism (Fig. 2). The second clade, the *carolinensis* clade, evolved a distinct developmental strategy not observed elsewhere in the genus to reach extreme levels of craniofacial dimorphism. The explicit use of phylogeny and comparative methods was critical to diagnosing the ancestral and derived developmental patterns. These methods allowed Sanger *et al.* (2013) to anchor the change specifically to the base of the *carolinensis* clade and specifically to the males. Without the use of a high-resolution map of developmental strategies, this detailed evolutionary scenario would not likely have been resolved. Many of the examples highlighted below have also capitalized on such a high-resolution approach.

It is breathtaking when we step back to consider the number of processes that unfold between the time of fertilization and sexual maturity. Within a given stage of organ development – initiation, pattern formation, or morphogenesis – a network of interacting molecules orchestrates cell movement, differentiation, proliferation, and death. As ontogeny elapses, new networks direct further differentiation of cells, tissues and organ growth. Phenotypic variation, the fodder of selection and adaptive evolution, can theoretically arise through subtle alterations at any stage of development. Furthermore, the environmental context of the developing organism can further alter the expression of phenotypic variation. The second and third pillars of our organismal framework are aimed at creating a more explicit focus on the processes that structure evolutionarily relevant variation, from its genetic basis through its expression within a given environment.

(2) The second pillar: understanding the origins of variation

Contemporary evolutionary biology is best equipped to examine the population-level processes that sort phenotypic variation and the macroevolutionary patterns of diversity that accumulated over time. By contrast, developmental biology is best fitted to examine the processes that generate relatively large-scale differences in morphology, such as the origin of major features or traits that are either present or absent. One of the strengths of developmental biology, which was readily adopted by Evo-devo, is its experimental rigour. However, relative to the ecologically relevant variation associated with adaptive evolution, the most widely used experimental tools of developmental biology are often too coarse to test how subtle differences in gene expression generate subtle quantitative variation that parallels population-level differences among individuals. The organismal framework strives to unite these perspectives better to understand the developmental origins of variation that is more akin to a

quantitative or ecological geneticist. Additional attention must shift towards examining quantitative variation among closely related species because this is where the greatest insights into the process of evolution and its relationship with ecology will be learned. When intra- and interspecific variation in development is analysed in the context of the first pillar, the organismal framework of Evo-devo has the potential to create a fully synthetic view of the evolutionary process that is unlikely to emerge when relying on coarse experimental techniques to compare developmental processes among distantly related taxa.

Evolutionary theory has consistently shown that many genes of small effect contribute to adaptive morphological variation (Roff, 2007; Rockman, 2012). At the same time, individual genes may be the foci of adaptation and have large effects. This observation is supported by both experimental and genetic association studies (e.g. Shapiro *et al.*, 2006; Manceau *et al.*, 2011; Conte *et al.*, 2012; Martin & Orgogozo, 2013; Gallant *et al.*, 2014). Whether this difference reflects a real or artificial chasm between theory and empirical research is another area where the organismal framework can offer new perspectives. Deeper insights into the evolutionary process will emerge once population geneticists and experimental geneticists collaborate. This is where we will learn the true origins of evolutionarily relevant quantitative variation.

Research on three-spined stickleback fish, *Gasterosteus aculeatus*, has illustrated the distinct explanatory power of combining population and experimental genetics. With the integration of developmental and population genetics Colosimo *et al.* (2005) discovered that the recurrent use of ectodysplasin (EDA), a secreted protein in the tumor necrosis family, has repeatedly been selected during the loss of lateral armour upon invasion of freshwater habitats. Colosimo *et al.* (2005) and Jones *et al.* (2012) established that this pattern was the result of repeated introgression and hybridization between marine and freshwater fish. Furthermore, the allele that is beneficial for life in freshwater is maintained in heterozygotes at an extremely low frequency (2.3% of fish) in marine habitats. This functional allele is a single nucleotide polymorphism of an *Eda* regulatory element that is responsible for the different armour phenotypes (O’Brown *et al.*, 2015). Specifically, variation of this nucleotide alters the sensitivity of *Eda* activity to Wnt signalling, and experimental alteration of this single nucleotide can recapitulate the associated armour variation (O’Brown *et al.*, 2015). This example illustrates the synthetic explanatory power – from molecules to morphology and from within-population variation to divergence across many populations – that can arise from an integrative approach to anatomical diversity.

Another realization that emerged through population-level Evo-devo is that quantitative trait evolution can arise from a step-wise *cis*-regulatory evolution process. Using African populations of *Drosophila melanogaster*, Rebeiz *et al.* (2009) were able to elucidate the amount and magnitude of impact of key nucleotide substitutions of the *cis*-regulatory region of a single gene, *ebony*, involved in

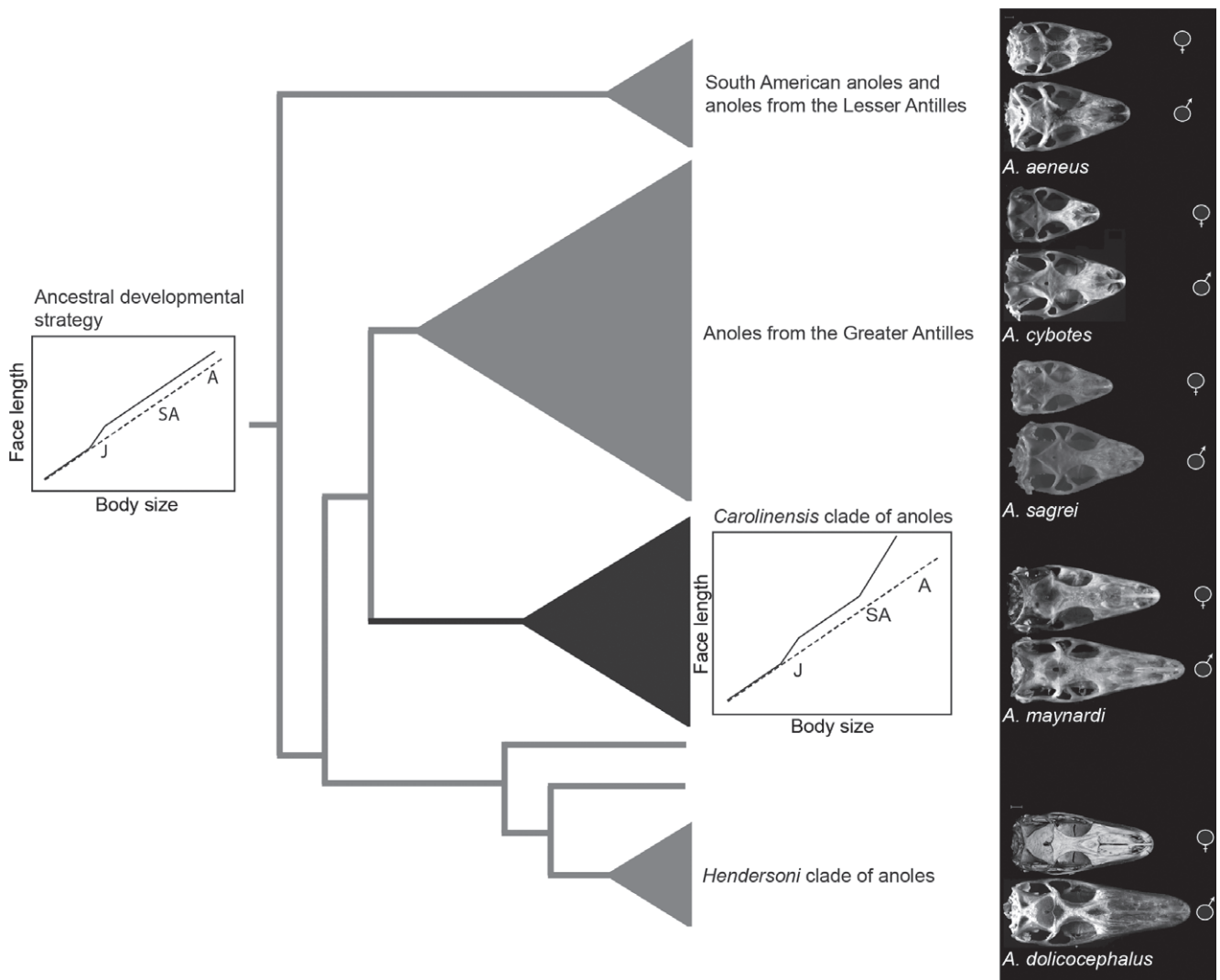


Fig. 2. Summary of the evolution of developmental strategies underlying craniofacial length dimorphism in *Anolis* lizards. Note that extreme levels of sexual dimorphism have evolved twice in anoles, in the *hendersoni* and *carolinensis* clades. The *hendersoni* clade reaches this level of dimorphism by exaggeration of an ancestral developmental strategy, sexual divergence during juvenile (J) growth (grey). The *carolinensis* clade has evolved a novel strategy not seen elsewhere in the genus (black). These anoles diverge as subadults (SA) entering sexual maturity and continue to diverge throughout adulthood (A). Figure modified from Sanger *et al.* (2013).

generating adaptive variation in pigmentation. In addition, they were able to determine the origin and chronology of emergence of this nucleotide variation, which arose through a combination of both standing genetic variation and mutations (Rebeiz *et al.*, 2009). One of the major implications of this work was that quantitative variation can arise through *cis*-regulatory changes in a gradual manner that further limits adverse effects of pleiotropy. This supports the widely held belief that regulatory evolution may yield more molecular pathways to adaptation (Carroll, 2008; Rebeiz *et al.*, 2009).

Focusing attention on quantitative variation in non-model species does not require abandonment of the experimental hallmarks of Evo-devo. It requires more refined tools. Experimental validation of candidate genes for quantitative variation may require experimental techniques that are

more nuanced than traditional knock-out studies that functionally eliminate a gene's expression from a species' developmental repertoire. In these quantitative cases, experimental validation may require dilution series of small molecule inhibitors (e.g. Parsons *et al.*, 2014), RNA interference (RNAi; e.g. Refki *et al.*, 2014), or hormones (e.g. Moczek & Nijhout, 2002) to recapitulate levels of gene expression observed in nature. Quantitative luciferase assays (e.g. Leal & Cohn, 2016) or pyrosequencing (Wang & Elbein, 2007) may be used to test the role of enhancer or allelic variants in driving expression differences (e.g. O'Brown *et al.*, 2015; Mallarino *et al.*, 2017). The precise techniques utilized and the limitations that come with them will depend on the system being studied, but the importance of experimentally validating candidate genes/pathways remains. When the quantitative strengths of Evolutionary Biology are combined

with the experimental rigour of Evo-devo, new insights about the evolutionary process are sure to emerge.

Evolutionary insights gained from *Peromyscus* mice exemplify the strengths of integrating population and experimental developmental genetics. Linnen *et al.* (2009) identified the molecular basis of pigmentation differences in *Peromyscus* populations living on different coloured substrates as population-specific isoforms of *Agouti* as the locus of evolution. Their population genetic analysis revealed that these isoforms likely arose *de novo* and were not due to standing genetic variation. Further analysis revealed that multiple mutations within *Agouti* are likely responsible for the entire spectrum of phenotypic effects observed among these populations, but their pleiotropic effects are minimized by the expression of those isoforms which are under the control of tissue-specific promoters (Linnen *et al.*, 2013). The developmental role of these functional alleles was later quantified and experimentally validated by Manceau *et al.* (2011) and Mallarino *et al.* (2016). Combined these authors demonstrated the role of *Agouti* in the developing hair follicle and that multiple populations have independently converged on the use of these isoforms to generate lighter pigmentation.

These examples illustrate how unique evolutionary insights can emerge from the integration of population thinking and Evo-devo. With the integration of an organismal perspective within Evo-Devo, the longstanding divide between the approaches taken within evolutionary and developmental biology will further narrow.

(3) The third pillar: dissecting organismal complexity

While the second pillar emphasized the need to elucidate the processes that generate evolutionarily relevant variation, the third pillar emphasizes the need to understand better the complexity of processes that structure variation. Phenotypic variation is not produced equally in all directions (Cheverud, 1982, 1996; Maynard-Smith *et al.*, 1985; Hendrikse, Parsons & Hallgrímsson, 2007; Hallgrímsson *et al.*, 2009). Therefore, phenotypic evolution can more easily proceed in certain directions compared to others. Furthermore, the expression of particular phenotypes and the degree of variation surrounding their average shape can be significantly impacted by the developmental environment. These ideas are contained within the conceptual silos of morphological integration, constraint, and phenotypic plasticity, respectively. However, to understand more thoroughly the ways that these processes interact to shape the rate or direction of morphological diversification we must not treat them as distinct areas of study examined in disparate species. Instead they must be incorporated into a synthetic understanding of morphological evolution within a diversifying clade. The organismal framework of Evo-devo is bringing new perspectives and clarity to these long-standing questions of evolutionary biology. Comprehensive reviews of each conceptual area are beyond the scope of this review. Here we emphasize their importance to the formulation of Evo-devo's organismal framework.

Evolutionary geneticists have long studied structured patterns of variation by describing patterns of correlations among phenotypic traits at the population level (i.e. the **P** and **G** matrices; Cheverud, 1988, 1996; Melo *et al.*, 2016). Evo-devo biologists now strive to understand the biological bases of those statistical patterns to gain deeper insight into the ways that the intrinsic properties of organisms can influence the evolutionary process. These attempts can be broadly categorized into two areas of study: morphological integration and modularity, and constraint. These are closely related conceptual areas (Klingenberg, 2008, 2010) that need to be considered independently when discussing their underlying biology. In each of these cases there remain conceptual difficulties when parsing apart pattern from process, which have clouded many years of study (Hallgrímsson *et al.*, 2009).

(a) Morphological integration and modularity

Morphological integration refers to correlations between multiple traits within an organism (Cheverud, 1982, 1996; Olson & Miller, 1999; Klingenberg, 2008; Melo *et al.*, 2016). Correlation among traits can arise from diverse processes including a shared developmental origin at the tissue level, shared gene expression, common response to growth or steroid hormones, or shared function where muscles interact with multiple skeletal elements (Hendrikse *et al.*, 2007; Mcglathlin & Ketterson, 2008; Hallgrímsson *et al.*, 2009). When traits are 'integrated' they will evolve in a concerted fashion. Integration can vary in its degree (strong or weak) and in its direction (positively or negatively correlated). Modularity refers to the pattern of integrated traits, where traits within modules are more strongly integrated than traits found in different modules (Cheverud, 1996; Wagner & Altenberg, 1996; Klingenberg, 2008; Melo *et al.*, 2016). Therefore, modules partition organisms into series of functionally or developmentally related traits.

Understanding the biological basis of integration and modularity will help predict what developmental processes are likely to contribute to adaptive evolution and which are likely to constrain the rate or direction of evolution. The biological bases of integration patterns are often inferred based on the correlation patterns observed in the adult form, not by the measuring of embryos or juveniles. This is particularly common in studies of vertebrate skull diversity, which consistently recover facial and neurocranial modules in mammals, lizards, and birds (e.g. Richtsmeier *et al.*, 2006; Sanger *et al.*, 2012b; Felice & Goswami, 2018). These studies often suggest that these modules represent the early embryonic division of neural crest and mesodermally derived skeletal elements. However, similar patterns could arise because of later processes overwriting those morphogenetic events including cartilage formation, brain and facial growth, and muscle–bone interactions (Hallgrímsson *et al.*, 2009). This creates a hierarchical series of correlated patterns, whereby modules that arise late overshadow our ability to clearly observe earlier patterns. The biological details of morphological integration and modularity will be elucidated

as we move beyond studies of adult structure and incorporate a more organismal perspective into research programs.

When selection acts on the adult form, the developmental and/or functional determinants of integration will establish how that species responds to selection. These biological determinants will dictate whether relatively few or many ontogenetic stages are affected by the change and how many traits are impacted by selection (Sanger *et al.*, 2011). Measuring individuals of different ages could address when integration patterns are established. Manipulating biomechanical loads throughout ontogeny (e.g. offering an animal hard or soft prey, changing locomotor performance) could address how flexible or rigid modularity patterns and integration intensity are to the organism. To our knowledge, these experimental approaches have not been widely employed. These analyses can be paired with studies of phenotypic plasticity or comparisons among different strains/populations (e.g. Jamniczky & Hallgrímsson, 2009; Parsons *et al.*, 2018) to understand better how integration affects the evolutionary process at microevolutionary levels and how this translates to macroevolutionary diversification if compared among closely related species. It is also possible for these manipulative experiments to be paired with new techniques looking at ‘anatomical networks’ that allow for simultaneous examination of modularity patterns in both hard and soft tissues (Esteve-Altava, 2017; Molnar *et al.*, 2017; Powell *et al.*, 2018).

(b) Constraint

The morphological diversity we observe in nature is not uniformly distributed across all theoretical possibilities. If we were to map morphological diversity in a multidimensional space, holes would exist that no species, extinct or extant, occupies. For at least the last 60 years, one of the fundamental questions has been: why do we see certain forms in nature and not others (Raup & Michelson, 1965; Raup, 1966; Gould, 1977; Alberch *et al.*, 1979; Alberch, 1980, 1989; Losos, 2011; Olson, 2012)? In recent evolutionary literature, non-random patterns of phenotypic variation are often discussed within the context of evolutionary constraints, which are thought to restrict or bias the possibilities of a diversifying clade. The short-term effect of constraints on a species’ response to selection is well established in both theory and field study (Cheverud, 1984; Arnold, 1992; Schluter, 1996), but there remains little consensus about the relative importance of constraint on macroevolutionary patterns of morphological diversity (Beldade & Brakefield, 2002; Beldade, Koops & Brakefield, 2002).

We define an evolutionary constraint as a process that affects the evolutionary response of a character to external selection acting at a focal life stage (Schwenk & Wagner, 2004). Therefore, developmental constraints represent population-level emergent properties of developmental systems rooted in the history of a clade. Just like anatomical traits, constraints have evolutionary histories of their own; they have origins and are eventually lost (Wagner & Müller, 2002; Schwenk & Wagner, 2004; Pavličev & Cheverud,

2015). Embracing the organismal perspective of Evo-devo promises to bring renewed vigour to discussions of whether intrinsic constraints shape macroevolutionary patterns of morphological diversity. By densely sampling developmental parameters among tetrapod vertebrates, several recent studies have effectively described how development may bias the ways in which species respond to selection. Young *et al.* (2014) demonstrated that divergence in amniote craniofacial morphology was limited to stages following fusion of facial prominences together to form the upper jaw, palate, and face of amniote vertebrates. They found that there is a high degree of phenotypic variation during both early patterning and late morphogenesis of embryonic cranial shape among species. Still, the intermediate developmental stages when fusion occurs create a developmental bottleneck. Experimentally changing the size or proportion of the prominences induces a high rate of clefting, presumably because the prominences are no longer in the proper position to fuse. Thus, the physical requirements associated with fusion of facial prominences constrain the potential to generate craniofacial variation to specific developmental time periods. The majority of amniote facial diversity is the result of processes that occur following fusion. The variation present prior to fusion is not associated with diversity and may represent neutral changes associated with developmental drift (True & Haag, 2001). Strikingly, Powder *et al.* (2015) described a similar scenario during the divergence of cichlid craniofacial proportions. This suggests that constraints associated with early craniofacial development may not be unique to amniotes. In spite of the inherent differences in craniofacial morphogenesis, post-morphogenetic divergence in craniofacial form may be a universal constraint among all vertebrates.

In these examples of craniofacial divergence, the constraint emerges when we consider the limited ways that amniotes can respond to selection. These studies reveal that despite the plethora of ways that diversity could theoretically be generated, developmental processes can restrict the options on which selection can work. Evolutionarily, relatively large-scale changes in facial morphology may require many transitional steps to occur. These constraints are tissue-level properties of the developmental system. It is unlikely that the addition of new mutations could modify these processes to alleviate this constraint without dramatic restructuring of the developmental system.

(c) Phenotypic plasticity

The environment in which an organism develops can have dramatic effects on its form. These effects are broadly categorized as phenotypic plasticity (Pigliucci, 2001; West-Eberhard, 2003). Although plasticity was once thought to be a relatively isolated phenomenon, more recent observations show that it is in fact widespread (Pfennig *et al.*, 2010; Moczek *et al.*, 2011; Gilbert & Epel, 2010). For developmental biologists, it is typical to control environmental parameters in order to dissect genetically the robust developmental pathways underlying the formation of a trait. Therefore, these studies tend to exclude the

dynamical role that ecological processes have in generating trait variation, although calls have been made to narrow this gap (e.g. Gilbert & Epel, 2010; Abouheif *et al.*, 2014; Gilbert, Bosch & Ledón-Rettig, 2015). For evolutionary biologists, while some have argued that plasticity would slow adaptive evolution by buffering heritable genetic variation from the effects of natural selection, others have argued that phenotypic plasticity can drive adaptive evolution and diversification (West-Eberhard, 2003; Pfennig *et al.*, 2010; Moczek *et al.*, 2011). We argue that embracing the organismal perspective to Evo-devo can shed new light on this controversy, particularly by discovering signatures of ancestral plasticity and genetic accommodation in the context of the model clade approach.

If plasticity is to have a lasting effect on the evolutionary trajectory of a lineage, the phenotypic variation produced or the shape of the plastic response must be hard-wired into the genome (West-Eberhard, 2003; Ehrenreich & Pfennig, 2016; Levis & Pfennig, 2018). The ability of a lineage to respond to environmental stimuli is not complete evidence in itself that plastic responses followed by genetic fixation play a role in phenotypic divergence, and therefore corroborative field work and natural observations describing nascent examples of plasticity-driven speciation would be illuminating. Because selection presumably acts directly on the plastic response and not directly on developmental processes related to morphogenesis of the focal trait (which could occur subsequently), observing a change in the degree of plasticity between ancestral and derived states is a critical step in testing hypotheses of plasticity-driven evolution (Levis & Pfennig, 2018).

To test the hypothesis more thoroughly that developmental plasticity fuelled the adaptive divergence of a trait, it will be important to compare the state of genetic networks in divergent species (or populations) involved in the development of a particular trait, in relation to ancestral plastic responses within the larger clade (Ehrenreich & Pfennig, 2016; Schneider & Meyer, 2017). This is where the well-established experimental tools of Evo-devo can make a significant contribution to this ongoing debate. Because of the pleiotropic nature of developmental genes, purely genomic and transcriptomic analysis will not offer the same level of explanatory power as an approach driven by this organismal philosophy of Evo-devo. Techniques such as tissue-specific RNA-sequencing and chromatin immunoprecipitation sequencing (ChIP-seq) can readily be employed across populations and species of non-model organisms, as well as across diverse environmental conditions, to elucidate the details of the transcriptional networks in species with different degrees of plasticity (e.g. Gunter *et al.*, 2013; Duncan, Gluckman & Dearden, 2014; Gibert *et al.*, 2016). *In situ* hybridization can be used to look at spatial-temporal changes in gene expression at the tissue level. More importantly, a breadth of experimental tools, readily accessible to non-model systems, including RNAi and clustered regularly interspaced short palindromic repeats (CRISPR), can be used for functional manipulation of gene expression to

test whether certain genes are either involved in modulating the plastic response itself or downstream patterning and growth changes involved in shaping morphological form (e.g. Xu *et al.*, 2015; Kijimoto & Moczek, 2016; Seroby *et al.*, 2016; Sieriebriennikov *et al.*, 2017). This battery of comparative and experimental techniques could provide unheralded insights into the evolution of plasticity and the role of genetic accommodation in morphological diversification.

Selection on genetic variation has clearly played an important role in the dramatic adaptive radiation of cichlid fish in East Africa (Kocher, 2004; Brawand *et al.*, 2014), yet adaptive plasticity is widespread and may have also contributed to their rapid diversification (Muschick *et al.*, 2011; van Rijssel *et al.*, 2015). Several experimental studies manipulating food resources resulted in plastically altered jaw morphologies phenocopying differences among species with alternative feeding methods (Meyer, 1987, 1990; Muschick *et al.*, 2011; Parsons *et al.*, 2014). These findings suggest that evolutionary shifts in jaw morphology could have been facilitated by plastically induced variation that is subsequently genetically fixed, if a particular jaw morphotype is consistently advantageous (Meyer, 1987). This example also illustrates the importance of incorporating behaviour into studies of plasticity. Without changes in an organism's behaviour, the plastic responses observed in jaw morphology would likely never have been induced.

Together, these examples, taken from various diverse taxa, illustrate the power of this unified three-pillar approach which lies at the core of the organismal framework of Evo-devo. Each example provides insights that would have likely been missed with more narrow approaches to the study of morphological diversity. Using these three pillars as a starting point, the incorporation of additional areas of organismal biology is readily feasible. For example, additional attention to the interaction of behavioural variation with the induction of alternative morphologies could be an important area to investigate the way that species respond to rapidly changing environments (Miner *et al.*, 2005; West-Eberhard, 2005; Snell-Rood, 2013). Similarly, sexual dimorphism, particularly the development of secondary sexual traits, also remains a relatively understudied area within Evo-devo (Williams & Carroll, 2009). This is another area where a holistic organismal perspective could generate large dividends. The organismal framework will be a useful starting point for these analyses.

III. OPERATIONALIZING THE ORGANISMAL FRAMEWORK

Mayr (1961) famously divided biological questions into those that address ultimate (evolutionary) and proximate (genetic or developmental) causations. Tinbergen (1963) better operationalized Mayr's distinction by posing four questions of biology (Fig. 3). Two of the questions are related to time and two are related to the current state of the trait. The time dimension of these questions is concerned with how the

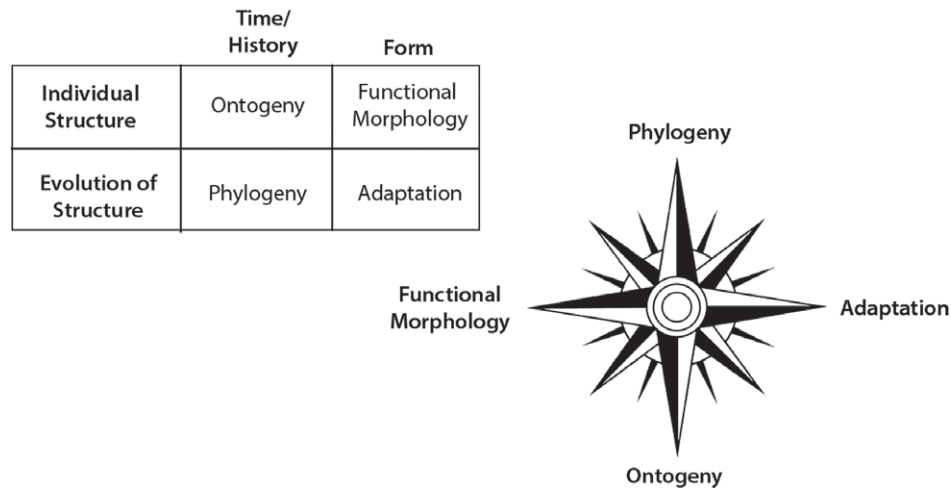


Fig. 3. Tinbergen's perspectives of a structure in (i) its current state: what is its function as related to its biomechanical properties and what is its utility/adaptive value; and (ii) as a function of time: what is the nature of its ontogeny and what is its evolutionary history. By incorporating Tinbergen's Compass into our research programs to navigate through these perspectives we can gain a more robust biological understanding of a trait and its variation.

trait is constructed during the developmental process and the evolutionary history of the trait. There are two questions regarding the current state of the trait, its structure and function. With regard to functional morphology, how does a structure work? Also, what is the adaptive significance of the trait (Nesse, 2013) or, in the case that the trait arose as a by-product independent of adaptation, what is its current utility (Bateson & Laland, 2013)?

Tinbergen has provided us with a heuristic framework to consider two major things that we are faced with as evolutionary developmental biologists and with the identity of the field: (i) what are the critical questions that are essential to understanding the biology of a trait, and (ii) how can these be integrated to form a complete biological understanding of form (Bateson & Laland, 2013). These four questions can be treated as specific lines of investigation and criteria that can guide, as a compass, research directions that each community working on a particular taxonomic group should consider and attempt to integrate (Fig. 3).

The compass also integrates well with the three supporting pillars of the organismal framework of Evo-devo. From north-to-south, we attempt to trace developmental transitions over evolutionary time within a model clade. From west-to-east, we can integrate our current understanding of the functional relevance of structural variation in the context of adaptation and utility of the trait. From this we can gain insight into the ecological relevance of trait variation and can understand better the fitness consequences of this variation. The northwestern quadrant is best aligned with an understanding of the transitions in structural and functional variation across species within a model clade, while the northeastern quadrante incorporates the model clade approach within an ecological context, enabling us to understand the evolutionary history of the trait alongside the enriching perspective of life-history and adaptive significance. By integrating the southwestern

quadrant of the compass, research programs can understand the genotype–phenotype map. Here we can effectively draw connections between biological levels where variation occurs, from the level of developmental processes to that of morphological structure and function. Finally, the southeastern quadrant focuses on finding associations between developmental variation, and ecological diversity, where we consider environmental variation as both a selective force as well as a source for developmental variation resulting from developmental plasticity. One does not have a comprehensive understanding of morphological evolution until all of the points of the compass can be addressed.

We are now beginning to understand how water strider legs develop, as well as a great deal about the relationship between how they work, their structural variations, the potential adaptive value of this variation and their evolutionary history. Therefore, the waterstriders form a preeminent example of the power of the organismal framework. It is through the integration of the perspectives around Tinbergen's compass where deeper insights, into the functional, phylogenetic, and adaptive significance of variation emerging from developmental processes will be found.

IV. FROM MODEL SPECIES TO MODEL CLADES: WATERSTRIDERS AS A PREEMINENT EXAMPLE OF THE ORGANISMAL FRAMEWORK

Waterstriders are a highly speciose and ecologically diverse group of semi-aquatic bugs (Spence & Anderson, 1994). A wealth of knowledge has accumulated regarding the ecological significance of several life-history characteristics of this group including habitat selection (Andersen, 1982), methods of dispersal (Vepsäläinen, 1978; Zera, Innes & Saks, 1983), plasticity related to climate change (Blanckenhorn &

Fairbairn, 1995), sexual dimorphism and sexual conflict (Fairbairn & Preziosi, 1994; Rowe *et al.*, 1994; Arnqvist & Rowe, 2002), as well as differential diapause and developmental timing (Vepsäläinen, 1978), many aspects that are central to the organismal framework of Evo-devo.

The most striking aspect of waterstriders is their ability to traverse water surfaces, through a diversity of incredible biomechanical innovations (Hu, Chan & Bush, 2003; Gao & Jiang, 2004), which have a profound influence on their behaviour, reproductive strategies and evolutionary diversification (Matsuda, 1960; Spence & Anderson, 1994). Research on waterstriders is a prime example of what can be elucidated when embracing the organismal framework, which is guided by Tinbergen's compass and anchored with a high-resolution model-clade approach. There is no traditional genetic or developmental model system as a close relative to waterstriders, yet researchers have made recent headway in developing extensive developmental genetic and genomic tools (Santos *et al.*, 2015). In addition to elucidating the developmental basis of various novel structures, from feather fans used for propulsion on the water surface (Santos *et al.*, 2017), and intricate sexually dimorphic antennal hooks for mating (Khila, Abouheif & Rowe, 2012), researchers on this group have discovered the transitional steps in the origin and elaboration of traits at multiple stages of life history (i.e. patterning, growth, and developmental allometry). Importantly, this work illustrates the complexity of modifications that may underlie trait diversification. Furthermore, it is likely that failure to examine this quantitative variation within the proper phylogenetic context would have missed the complexity of these evolutionary transitions.

For waterstriders, the transition from a terrestrial ancestor to open water coincided with a dramatic change in body plan. The terrestrial ancestor had a typical Hexapoda leg length relationship where the third pair of legs is longer than the second. By contrast, the waterstrider's second pair of legs is substantially longer than the third. This facilitated the use of the second pair of legs as oars and the third pair as rudders to glide on the water surface. An integrative approach of developmental genetic and phylogenetic investigation uncovered that this change in body plan and transition to the water surface occurred through gradual evolutionary steps facilitated by transitional developmental genetic changes (see Fig. 1). During embryonic development, the second pair of legs grow more than the third (Khila, Abouheif & Rowe, 2009). In the ancestral terrestrial bugs, the spatial expression of the highly conserved homeobox (*Hox*) gene, *Ubx*, is normally restricted to the third pair of legs and is known to elongate them (Stern, 2003; Mahfooz *et al.*, 2007). By contrast, spatial expression and function of *Ubx* is completely different for waterstrider leg development: *Ubx* is expressed in the second and third pair where it elongates the second and has evolved a novel function to shorten the third (Khila *et al.*, 2009; Khila, Abouheif & Rowe, 2014). *Ubx* is capable of these opposing functions due to tissue-specific expression levels and sensitivity, and specific downstream effector gene expression

(Refki *et al.*, 2014; Armisen *et al.*, 2015). The properties of *Ubx* in the second and third pair of legs facilitated both the allometric difference in size between legs and between segments within each leg (Khila *et al.*, 2014; Refki *et al.*, 2014). *Ubx* carries out this growth function independent of its critical patterning role, but also through the growth function of downstream patterning genes (Refki *et al.*, 2014). The most important outcome of this work emerges from the tracing of the relationship between these developmental genetic properties and leg morphology variation across the group.

If one had only compared the highly derived waterstrider to its terrestrial ancestor (as with the traditional approach to Evo-devo, Fig. 1), one would have misunderstood the process by which developmental pathways and morphological structures evolved in this clade. By taking a high-resolution phylogenetic approach, it was determined that the evolution of this complex relationship between *Ubx* and morphological form is the product of several transitional steps and not an abrupt novel change (Fig. 4). Specifically, it was shown that for transitional species that are both terrestrial and aquatic, the relationship between leg lengths remains ancestral, yet the expression of *Ubx* and its function to elongate has already moved to the second pair of legs. Although it is predicted that an increase in leg length would result in a decrease in leg movement frequency these transitional species evolved the capacity to employ alternative locomotion strategies on the two surface substrates (Crumiere *et al.*, 2016). These species simultaneously increased leg frequency and leg length, facilitating fast speed on the water surface. Therefore, the function of *Ubx* in elongating the second pair of legs preceded its function in shortening the third pair of legs, which arose afterwards with the novel waterstrider body plan (Khila *et al.*, 2014). Finally, it was demonstrated that this leg allometry found in the Gerridae group independently evolved in the group Velidae through similar function of *Ubx* in the overall shortening of the third leg but the function of *Ubx* in the quantitative sizing of each leg segment evolved differently between these groups (Khila *et al.*, 2014).

Within waterstriders, functional developmental genetic techniques have been combined with functional morphology and behavioural assays to discern the consequences of developmental modifications on trait variation in the context of fitness and adaptation. In other words, rather than just assessing phenotypes, manipulated embryos were put through a battery of tests to assess the performance consequences of their induced changes. Armisen *et al.* (2015) functionally demonstrated that *Ubx* acts through the gene *gilt* in order to establish the biomechanically relevant differences between the second and third pair of legs. By quantitatively manipulating this gene, which was identified using tissue-specific comparative transcriptomics (not a candidate gene approach), individuals developed shorter legs, which resulted in decreases in both jumping ability and predator evasion. Together, this integrative approach, which aims at reconstructing the origin and elaboration of morphologies, reveals the transitional developmental genetic

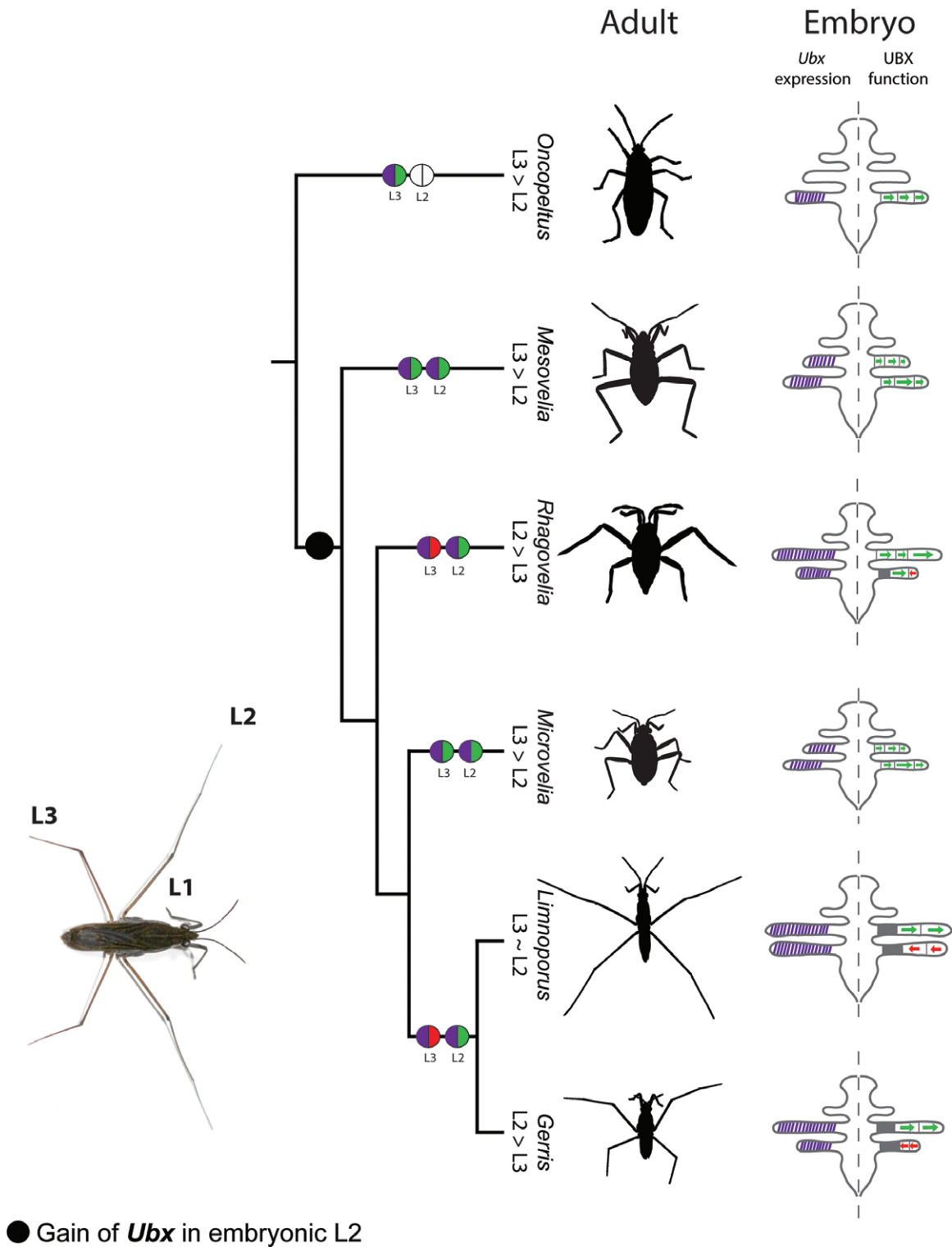


Fig. 4. Summary of the evolution of developmental transitions underlying morphological transitions in waterstrider leg diversification. By taking a high-resolution model-clade approach, Khila *et al.* (2009, 2012, 2014) have demonstrated that the dramatic diversification of leg morphology in *Gerris* is the result of several changes in *Ubx* expression in both space and time, as well as modifications in its function from its ancestral growth-promoting role to a novel dual growth repressor/activator role. In addition, this approach further demonstrates that similar leg size relationships can be obtained through different patterns of leg segmentation growth regulation of *Ubx*. Left-half of circles represent *Ubx* expression (purple), right-half represent UBx function (green, positive growth regulator; red, negative). Left side of embryo depicts expression domain of *Ubx* (purple), right side depicts function in each segment (green, positive; red, negative). L1, first pair of legs; L2, second pair of legs; L3, third pair of legs.

steps which facilitated transitions in morphological variation and function that coincide with the ecological transitions these organisms encountered historically (Santos *et al.*, 2015).

Waterstriders highlight multiple lessons from the organismal framework. First, the model clade approach highlighted the discordance between developmental and morphological evolution. Furthermore, the stage- and segment-specific expression patterns, in parallel to the independent evolution of segment length, highlights the modular nature of leg development. Without the organismal approach one may have predicted that the shared expression of *Ubx* would constrain limb length evolution. Instead, this illustrated that the changing role of *Ubx* may have facilitated the evolution of waterstriders into a new habitat. By experimentally manipulating the development of these structures one can gain a fuller picture of what phenotypic variation is possible and what is not. It also gives us the ability to assess the function of trait proportions not observed in nature to better assess the adaptive value of subtle changes in limb length. Different variants can be tested in a hydrofluidics/locomotion context to see which are incapable of adapting to the water surface. To date, there is a great deal unknown regarding the contribution of plasticity to leg body plan diversity. It is possible that growth pathways that are sensitive to environmental variation may contribute to the differential growth of the legs and their segments. Blanckenhorn & Fairbairn (1995) described changes in developmental timing, rates of growth and differences in body size in a species of waterstrider (*Aquarius remigis*) resulting from seasonal variation along a latitudinal cline. Interestingly, they also compared the mid-femoral length of the leg across populations. Variation in this segment had previously been described as a suitable marker to differentiate populations morphologically within this species and they found that Northern populations had longer segment length than their southern counterparts. It would be interesting to look, in finer detail, at whether the degree of developmental plasticity of *A. remigis* leg lengths (and leg segments) changes across these latitudinally striated populations, and to determine what developmental pathways facilitate these changes. More generally, how flexible is this plastic response, from the different populations, to climate change and how has this contributed to the diverse leg body plans observed across species?

V. THE WAY FORWARD IS THROUGH A MORE BALANCED APPROACH TO EVO-DEVO

The organismal framework of Evo-devo that we discuss herein is not a critique of the success this field has seen in recent decades. On the contrary, we feel that the most productive way for Evo-devo to move forward will be through a more balanced approach between the traditional and organismal approaches. Organismal systems are best suited to address the evolutionary mechanisms underlying the diversification of complex morphological systems while

the traditional model systems are best employed for a deeper analysis of regulatory mechanisms on a larger scale. By embracing the distinct strengths of these two approaches, the field is likely to find new areas of synthesis that would otherwise remain hidden. We hope that by formalizing these aspects of an organismal framework researchers will broaden the scope of their studies into new areas, encouraging interdisciplinary studies of morphology.

Compared to the traditional approach to Evo-devo whose focus is to uncover the molecular differences between two distinct phenotypes in increasingly fine detail, the organismal perspective strives to generate a high-resolution map of developmental changes that underlie transitional evolutionary modifications in morphology (Fig. 1). Furthermore, the organismal perspective draws explanatory power from the explicit integration of an organism's evolutionary history, the number of ways that phenotypic variation can be generated and sorted, and an understanding of the population-level processes that can scale to macroevolutionary differences. We feel that the strongest way forward for the field is to integrate more fully the strengths of experimental and comparative approaches while acknowledging their inherent limitations.

Model organisms provide unheralded insights into the molecular mechanisms of evolutionary change, yet they have a limited ability to address the process of adaptive morphological diversification. By contrast, non-model organisms may never have the technical power to functionally dissect developmental process to fine genetic detail, but they provide great evolutionary insights when used in the appropriate comparative context. The cost of sequencing now allows for non-model genomes, tissue-specific transcriptomes, methylomes, and enhancer diversity to be uncovered, thereby expanding the tractability of model clades for thorough mechanistic investigation.

Tschinkel & Wilson (2014, p. 442) stated, 'The story of any species chosen at random is an epic, filled with mysteries and surprises that will engage biologists for generations to come.' The organismal framework of Evo-devo has made, and is poised to make, further advances in conceptual areas that have long vexed the fields of Evolutionary and Developmental Biology. More generally, by placing the organism at the centre of our Evo-devo research programs, we will be able to pave the way towards a more integrative understanding of the 'epics of nature'.

VI. CONCLUSIONS

(1) The organismal framework of Evo-devo is providing rich insights into the evolutionary processes of morphological diversification. By taking a high-resolution model clade approach, we can go beyond comparative developmental biology at the level of model organisms to a level that traverses evolutionary scales (pillar 1). The explicit use of phylogeny and comparative methods will facilitate the

tracing of transitional modifications to development and morphology.

(2) Variation is the fodder of evolutionary change, yet evolutionary and developmental biology have traditionally studied dramatically different scales of variation using dramatically different approaches. Bringing new and directed attention to the processes that generate (pillar 2) and structure (pillar 2) patterns of variation the organismal framework of Evo-devo will bridge this long-standing divide between the fields.

(3) The perspectives detailed in this article are already alive and prospering within modern Evo-devo. More thoroughly integrating the organismal framework with the experimental approach to Evo-devo promises to shed new light on the evolutionary processes of morphological diversification. By placing the organism at the centre of our Evo-devo research programs, we will be able to pave the way towards a more integrative understanding of the ‘epics of nature’.

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